A METHOD TO PREVENT GRAFT REJECTION USING TGF-β TO INDUCE T SUPPRESSOR CELLS

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S.S.N. 09/833,526, filed April 11, 2001, which claims benefit under 35 U.S.C. § 119(e) to application Serial No 60,196,446, filed April 11, 2000, the disclosures of which are incorporated herein by reference in their entirety.

FIELD OF THE INVENTION

The field of the invention is related to compositions and methods useful for preventing graft rejection in a recipient following organ transplantation.

BACKGROUND OF THE INVENTION

Organ transplantation has been used to improve the quality of human life. Substantial progress has been made in the transplantation of kidneys, hearts, lung, livers and pancreas. Current immunosuppressive drugs are generally effective in blocking the immediate rejection of these organs. However, when the organ is from an unrelated donor, i.e., allograft, these drugs become less successful with the passage of time because immunosuppressive drugs are often ineffective in blocking chronic allograft rejection. In addition, there are significant side effects associated with long term immunosuppressive therapy. Each year approximately 10,000 kidney transplants are performed in the United States. While the chances that the graft will function well for at least one year have been increasing, there has been a lack of progress in preventing chronic allograft rejection during the past 20 years (See Figure 1; In Fundamental Immunology, 4th ed., Paul,.W.E. (ed.), Lippincott-Raven, Philadelphia, 1999, p.1201). As a result, only 50% of transplants are still functioning years later. There is an urgent need, therefore, for new methods to prevent chronic rejection.

Graft rejection occurs when the immune system of the recipient recognizes foreign histocompatibility antigens. Infrequently, rejection is caused by antibodies, either preformed or the result of multiple blood transfusions. Rejection generally occurs when T lymphocytes from the recipient recognize and respond to donor histocompatibility antigens (Pescovitz MD, Thistlethwaite JR Jr, Auchincloss H Jr, et al. J Exp Med 1984;160:1495–1508).

There are two major histocompatibility complex (MHC) loci. Both major and minor histocompatibility antigens have been described as well as the genes that encode them. One encodes MHC class I antigens which are recognized by CD8+ T cells and another encodes MHC class II antigens which are recognized by CD4+ cells. MHC class I antigens are expressed on almost all tissues of the body.

Both MHC I and II antigens are very polymorphic so that it is highly unlikely that antigens from unrelated individuals will be identical.

Differences in MHC antigens between donor and recipient trigger a strong immune response by the recipient which results in the rejection of the transplanted organ. Foreign MHC antigens are directly recognized by the recipient's immune cells and also indirectly recognized by antigen-presenting cells of the recipient which have processed donor MHC antigens. The classical model of allograft rejection emphasizes CD4+ T cells of the recipient recognizing MHC class II antigens of the donor. These activated CD4+ cells serve as helper cells for recipient CD8+ which are sensitized by direct recognition of donor MHC class I antigens. The activated CD8+ cells then kill donor cells by lysing them (Mizuochi T, Golding H, Rosenberg AS, Glimcher LH, Malek TR, Singer A. J Exp Med 1985;162:427–443. 205). Further studies have revealed additional participation of recipient antigen presenting cells, B cells, NK cells and NK T cells which adds complexity to the mechanisms responsible for graft rejection.

Graft destruction which occurs within the first few weeks after transplantation is called "acute rejection". Usually, the use of immunosuppressive drugs temporarily prevents this result. Unfortunately, the grafts may eventually fail weeks or months later. This failure is referred to as "chronic rejection." Both humoral and cellular mechanisms have been implicated in chronic rejection. Anti-donor antibodies have been claimed to promote chronic rejection, but this is controversial. It is generally believed that chronic rejection is the consequence of persistent sensitization of the immune system to donor MHC antigens. The immune cells of the recipients cannot "learn" to accept the donor MHC antigens as self and respond by attacking the graft.

There are two approaches to prevent graft rejection. The first is by treatment with non-specific immunosuppressants and the second is to induce donor-specific tolerance.

The standard first approach is to use immunosuppressive drugs such as steroids, azathioprine, mycophenolate, cyclosporine, FK-506, rapamycin, leflunomide, or 15-deoxyspergualin. These drugs suppress immune responses by inhibiting lymphocyte gene transcription, cytokine signal transduction, nucleotide synthesis and cell differentiation. These drugs are associated with lifelong increased risks of infection and malignancy. In addition, anti-T cell antibodies such as anti-lymphocyte serum or anti-thymocyte globulin are also powerful immunosuppressants. However, they have major side effects include serum sickness and infectious complications. More recently, OKT3, a mouse antibody directed against the CD3 antigen of humans, has become widely used in clinical transplantation. (Cosimi AB, Burton RC, Colvin RB, et al Transplantation 1981;32:535–539). Other monoclonal antibodies used include the antibody to the IL-2 receptor (anti-CD25) and the anti-ICAM-1 or anti-TNF-α to block the effector mechanism of graft rejection. These monoclonal antibodies also have broad toxic side effects.

The ultimate goal of transplantation immunology is to enable the recipient to become tolerant to donor histocompatibility antigens. That is, to prevent the recipient's immune cells from recognizing donor

antigens (i.e., accepting the donor organ as "self") so that the graft is not rejected. The current state of the art in this area is reviewed herein and elsewhere (See Hugh Auchincloss, Jr., Megan Sykes, and David H. Sachs In Fundamental Immunology, 4th ed., Paul, W.E. (ed.), Lippincot-Raven, Philadelphia, New York, 1999 pp 1182-1222).

Tolerance can be achieved by three mechanisms. The first is "clonal deletion"; the elimination of lymphocytes which react to the donor antigens. The second is "clonal anergy"; the failure of T cells to proliferate in response to donor antigen. Anergy is generally reversible and can be reversed by infection or elimination of antigen (Rocha B, Tanchot C, Von Boehmer H. J Exp Med 1993;177:1517–1521) (Ramsdell F, Fowlkes BJ. Science 1992;257:1130–1134). The third is "suppression"; which can be either non-specific or antigen-specific. Non-specific suppression can result from the secretion of soluble molecules that inhibit immune function. Suppressive molecules include prostaglandins (Snijdewint FGM, Kalinski P, Wierenga EA, Bos JD, Kapsenberg ML. J Immunol 1993;150:5321–5329; Betz M, Fox BS. J Immunol 1991;146:108–113), nitric oxide (Langrehr JM, Dull KE, Ochoa JB, et al. Transplantation 1992;53:632–640), and cytokines (Verbanac KM, Carver FM, Haisch CE, Thomas JM. Transplantation 1994;57:893–900); Raju GP, Belland SE, Eisen HJ. Transplantation 1994;58:392–396).

Certain T cells, called "suppressor cells", produce inhibitory cytokines which include IL-4, IL-10, and TGF-β which, non-specifically, block graft rejection (Qin L, Chavin KD, Ding Y, Woodward JE, Favaro JP, Lin J, Bromberg JS. Ann Surg 1994;220:508–519); (Qin L, Chavin KD, Ding Y, et al. J Immunol 1996; 156:2316 –2323); (Zheng XX, Steele AW, Nickerson PW, Steurer W, Steiger J, Strom TB. J Immunol 1995;154:5590–5600). The existence of alloantigen-suppressor cells have been reported (Pearce NW, Spinelli A, Gurley KE, Hall BM. Transplantation 1993;55:374–379; Roser BJ. Immunol Rev 1989; 107:179–202; Tomita Y, Mayumi H, Eto M, Nomoto K. J Immunol 1990;144: 463–473), but these cells are difficult to clone (Koide J, Engleman EG. J Immunol 1990;144:32–40).

Naturally occurring suppressor T cells produced by the thymus have been characterized in mice. These are CD4+ cells that express CD25, cell surface IL-2 receptor α chains (Shevach, E. A. (2000) Annu. Rev. Immunol. 18:423-449.; Seddon, B., and D. Mason, (2000) 21:95-99.; Sakaguchi, S., N. Sakaguchi, M. Asano, M. Itoh, and M. Toda, (1995) J. Immunol. 155:1151-1164). To date, this T cell subset has not been well described in humans and whether these cells can be expanded in the periphery is unknown.

CD4+ cells repeatedly stimulated with IL-10 or activated with immature dendritic cells develop down-regulatory activity (Groux, H., A. O'Garra, M. Bigler, M. Rouleau, S. Antojejko, J. E. De Vries, and M. G. Roncarolo, (1997) Nature. 389:737-742; Jonuleit, H., E. Schmitt, G. Schuler, K. Jurgen, and A. H. Enk. (2000) J Exp Med 192:1213-1222). These T cells, called TR1 or TR1-like cells, are anergic and their immunosuppressive effects are mediated by IL-10 and TGF-β. Anergic T cells suppress other T cell responses by targeting antigen-presenting cells (Taams, L. S., A. J. M. L. van Rensen, M. C. M. Poelen, C. A. C. M. van Els, A. C. Besseling, J. P. A. Wagenaar, W. van Eden, and M. H. M. Wauben (1998) Eur J Immunol 28:2902-2912; Vendetti, S., J. G. Chai, J. Dyson, E. Simpson, G. Lombardi and

R. Lechler, (2000), J. Immunol. 165:1175-1181). Unfortunately, large numbers of these cells are required for suppressive activity and their capacity to expand is very poor.

The profile of cytokines produced by T cells can affect the survival of an organ graft. The shift in T cell response from the pro-inflammatory Th1 response (IL-2 and IFN-γ) to the anti-inflammatory Th2 (IL-4, IL-10) response has been associated with allograft acceptance (Roser BJ. Immunol Rev 989: 107: 179–202; Lancaster F, Chui YL, Batchelor JR. Nature 1985; 315:336–337; Wilson. Immunol Rev 1989;107:159–176). Only limited data, however, implicate an active role of Th2 cells in tolerance induction (Bucy RP, Li J, Huang GQ, Honjo K, Xu XY.[Abstract]. FASEB J 1995;9:A497; Wilson. Immunol Rev 1989;107:159–176). Moreover, in some cases Th2 cells can mediate or contribute to graft rejection.

Methods to specifically direct T cells to become tolerant or produce inhibitory cytokines would be very helpful in promoting the survival of transplanted organs. Several tolerance-inducing strategies have been attempted in combination with conventional immunosuppressive drugs. In rodents, tolerance can be achieved by giving a dose of lethal irradiation to a mouse, and saving the animal by giving back T cell-depleted syngeneic and allogeneic bone marrow cells. The hematopoietic cells that repopulate the animal will display histocompatibility antigens of both donor and recipient cells and, therefore, will be tolerant to grafts from each mouse strain (Singer A, Hathcock KS, Hodes RJ., J Exp Med 1981; 153:1286). Non-myeloablative conditioning regimens have been described where mice are sublethally irradiated, T cell depleted with monoclonal antibodies and given either anti-CD154 or CTLA4Ig which block co-stimulatory molecules (Wekerle, 1999). This strategy achieves central tolerance and should have long lasting effects, but has not yet been performed in large animals. None of these strategies have been used to replace chronic therapy in clinical transplantation.

Peripheral tolerance can be achieved by blocking co-stimulatory molecules. The combination of CTLA4Ig and anti-CD154 markedly prolongs the survival of primary skin allografts in mice (Larsen CP, Elwood ET, Alexander DZ, et al. Nature 1996;381:434–438; Kirk AD, Harlan DM, Armstrong NN, et al. Proc Natl Acad Sci U S A 1997; 94:8789–8794). A major problem with strategies to block co-stimulatory molecules is that they cannot prevent generation of new T cells in the recipient capable of recognizing donor antigens.

There are examples of solid organ transplants that have survived for many years in human recipients who did not receive hematopoietic cell transplants (Starzl TE, et al. (1993) Transplantation, 55:1272–1277). In these instances, passenger leukocytes from the graft might emigrate to the thymus and tolerize subsequently developing thymocytes. The heavy doses of immunosuppressive drugs used to prevent acute rejection blocks this thymic education. Strategies to reduce the dosage of immunosuppressive therapy might overcome this problem and lead to long lasting central tolerance.

An ideal strategy to prevent graft rejection would be to induce T cells to develop the capacity to suppress the immune attack by the recipient against donor histocompatibility antigens. Although CD4+ cells repeatedly activated in the presence of IL-10 develop potent suppressor activity, these

cells have a very short life span and poor proliferative potential (Groux H, et al., (1997) Nature, 389:737-42). Thus, there is a need for a method to generate suppressor T cells which are hardy and able to proliferate.

SUMMARY OF THE INVENTION

In accordance with the objects outlined above, the present invention provides compositions and methods that can be used to induce T cell tolerance in a solid organ transplant recipient. The compositions include compounds that inhibit or suppress immune function by inducing a population of T cells to develop suppressor activity. Compounds useful in the compositions of the invention include anti-inflammatory cytokines, such as IL-4, IL-10 and TGF-β, chemokines, prostaglandins and nitric oxide.

In an additional aspect, the present invention provides methods of inhibiting graft rejection in a recipient comprising removing peripheral blood mononuclear cells (PBMC) from a donor and recipient, culturing the donor and recipient cells together in the presence of a compound that induces T cell suppressor activity, and administering these treated cells to the recipient following graft transplantation.

In a further aspect, the invention uses closed systems for the purification, conditioning and expansion of T cell populations before administering them to a patient.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the lack of progress in preventing chronic rejection over the last twenty years.

Figures 2A-C depict the effect of TGF-β on T cell subsets in the generation of cells that suppress cytotoxic T cell activity. One way allogenic mixed lymphocyte reaction (MLR) is used to generate regulatory T cells that suppress cytotoxic T cell activity. In allogeneic MLR, T cells from one individual (i.e., A) are mixed with cells from an unrelated individual (i.e., B). In the absence of regulatory T cells which suppress cytotoxic T cell activity individual A's T cells recognize B's cells as foreign and develop the capacity to kill B's cells.

Regulatory T cell subsets that prevent this killing are generated from A by negative selection by staining the cells with appropriate monoclonal antibodies and using immunomagnetic beads to remove the stained cells. The resulting T cell subsets are cultured for five days with cells from B in the presence of TGF-β. Controls consist of T cells cultured with stimulator cells in the absence of TGF-β.

The effect of TGF-β conditioned T cell subsets on allogeneic MLR was determined by mixing regulatory cells to effector cells in a ratio of 1:4. After five days of culture with stimulator cells from B, the cytotoxic activity of T cells from A was determined using a standard chromium release assay in which effector cells from A were mixed with chromium labeled lymphoblasts from B in the effector to target cell ratios shown. Chromium release was measured in a 4 hour assay (open squares). Control

T cell subsets cultured with stimulators in the absence of TGF-β are indicated by open circles. T cell subsets cultured with stimulator cells in the presence of TGF-beta are shown as closed circles. In all experiments, TGF-beta enhanced the capacity of the T cell subsets to suppress the generation of cyotoxic activity.

Figures 3A-3B depict two independent experiments showing the regulatory effects of TGF-β on CD4 CD45RA T cells cultured with stimulator cells in the presence or absence of TGF-β. Control CD4 CD45RA cells cultured with stimulator cells in the absence of TGF-β have modest to moderate suppressive activity. In contrast, T cells primed with TGF-β 1 ng/ml markedly suppress or abolish allo-CTL activity.

Figures 4A-4B depict that CD4 suppressor cells require cell contact to inhibit cytotoxic T cell activity. In these experiments, regulatory CD4 cells from CD4 CD45RA were conditioned with TGF-β as described in Figures 2A, 2B, 3A and 3B. An aliquot of the cells was mixed with responder T cells from A and stimulator cells from B. A separate aliquot of regulatory cells was separated from the responder and stimulator cells by a membrane. The results indicate that suppressor T cells require cell contact to exert their regulatory effects.

Figure 5 depicts suppression of lymphocyte proliferation by regulatory CD4+ T cells induced with TGF-β. Naïve CD4+ T cells from A were mixed with stimulator cells as described above and added to fresh responder and stimulator cells at the indicated ratios. The bars show the mean uptake of tritiated thymidine ± SEM after 7 days of culture. The lightly shaded bar (Nil) indicates the proliferative response of the responder T cells without added CD4+ cells. The darkly shaded bar indicates the effect of control CD4+ cells cultured with stimulator cells, but without TGF-β. The black bar indicates the effect of CD4+ cells cultured with stimulator cells in the presence of TGF-β (1 ng/ml). The effect of these CD4+ cells on the proliferative response of fresh responder cells added to irradiated stimulator cells after 7 days of culture is shown.

Figures 6A-6B depict the potency of CD4+ regulatory T cells induced by TGF-β and shows that they express CD25. Naive CD4+ T cells primed with irradiated allogeneic stimulator cells ± TGF-β (1 ng/ml) and CD4 regulatory cells were separated into CD25+ and CD25- fractions by cell sorting. Figure 6A depicts the effect of primed CD4+ cells mixed with fresh T cells at a 1:4 ratio. The suppressive activity is concentrated in the CD25 fraction. Figure 6B depicts the effect of various dilutions of these primed CD4+ T cells added to fresh responder cells on the generation of CTL activity. Results shown were performed at an effector to target cell ratio of 100:1. Significant suppressive activity was present when less than 1 CD4reg was mixed with 100 responder T cells.

Figures 7A-7B depict the effects of a regulatory composition on the expression of CD25 and CTLA-4 by CD4+ cells. The T cell subset was prepared from the spleens of DBA/2 mice. The CD4+ cells were cultured with irradiated allogeneic splenic lymphocytes from C57BL/6 mice with and without TGF-β (0.1-1ng/ml) for the days shown. The cells were stained for CD25 or CTLA-4 and the

percentage of cells expressing the respective markers was determined by flow cytometry. By day 5 the presence of TGF-β significantly enhanced expression of CD25 and CTLA-4.

Figures 8A-8B depict the proliferative response of CD4+ regulatory T cells to alloantigens. Naive CD4+ T cells and irradiated stimulator cells ($1x10^6$/ml) were cultured ± TGF-β for 5 days in serum free medium to generate CD4+ regulatory T cells. The cells were washed and rested for 3 days in culture medium containing 10% normal human serum. The cells were labeled with carboxyfluorescein (CFSE) and re-stimulated with irradiated allogeneic stimulator cells. The intensity of CFSE staining after three and five days 5 days of culture is shown. The heavy line shows CD4 regulatory cells and the thin line CD4+ cells primed with alloantigens without TGF-β. The proliferative response of the CD4+ cells primed with TGF-β is stronger than that of control CD4+ cells. Similar results have been obtained in four other experiments.

DETAILED DESCRIPTION OF THE INVENTION

The present invention makes possible the transplantation of solid organs such as kidneys, heart, lungs, liver and pancreas in humans using methods to prevent chronic graft rejection. By mixing large numbers of T cells *ex vivo* with a compound that induces suppressor activity, a population of suppressor or regulatory T cells is produced. Suppressor cells can be administered to a recipient before, at the time of, or after the transplant to prevent the recipient's T cells from killing donor cells, thereby inducing tolerance and long term survival of the transplant. The particular advantage of this procedure is that the immune system of the recipient is harnessed to perform a function that conventional and experimental therapies have failed to achieve.

Thus, the present invention prevents graft rejection by suppressing T cell activation and inducing a tolerant state in the recipient's cells. This is achieved by inducing some of the recipient's cells to assume a surveillance role and prevent other recipient cells from mounting an immune attack against the graft. The net effect is for the recipient's lymphocytes to become tolerant of the histocompatibility antigens of the donor, thereby making possible the long term survival of the graft.

This strategy is unlike almost all other treatment therapies currently in use because cells, rather than organ recipient as a whole, are treated with potent pharmacologic agents. Thus, the recipient is spared from the severe side effects associated with these agents.

An advantage of the present invention is that it may reduce or minimize the need to administer highly toxic immunosuppressive medicines that must be given to recipients of organ transplants. In the present invention, the recipient's own immune cells are induced to suppress the immune response, thus, the doses of toxic immunosuppressive drugs administered for this purpose can be reduced.

Thus, the present invention provides a lowered probability of serious side effects as only trace amounts of compounds which may block the immune response are returned to the recipient.

The present invention shows that a population of a recipient's T cells can be induced to block an immune attack against the donor organ. Without being bound by theory, it appears that there are

several ways the methods of the invention may work, either alone or in combination. First, recipient cells may be activated to become tolerant to the donor cells. Second, recipient cells may be activated to assume a surveillance role and prevent other recipient cells from killing donor cells. Third, treatment of recipient cells with a suppressive compound may inhibit the cytotoxic activity of some recipient T cells by inducing other recipient cells to have suppressor activity. For example, in Figures 2-4, TGF-β is used to induce various T cell subsets to have suppressor activity and prevent lysis of donor cells. Suppressing the lysis of donor cells by recipient cells will decrease or eliminate chronic graft rejection.

Accordingly, the present provides compositions and methods of inducing T cell tolerance in an organ transplant recipient. The present invention provides methods comprising removing peripheral blood mononuclear cells (PBMCs) from both a recipient and a donor, mixing the recipient and donor cells together, and treating the cells with a regulatory composition to generate a population of suppressor T cells. These suppressor T cells can be introduced into a recipient at the time of transplant, or at various times thereafter to prevent chronic graft rejection.

By "recipient" herein is meant a human which is to receive an organ transplant. In some case, the recipient may be an animal, including but not limited to rodents including mice, rats, and hamsters, domestic animals, wild animals and primates. Likewise, for the purpose of the invention, a "donor" is a human or animal from which the organ is obtained.

The present invention is directed to methods utilized in organ transplant. By "organ" herein is meant solid organs such as kidneys, heart, lungs, liver, and pancreas.

In a preferred embodiment, rejection of the transplanted organ is prevented by inducing tolerance in a recipient's T cells by administering T cells conditioned to become suppressor cells. By "tolerance" or "T cell tolerance" or grammatical equivalents herein is meant immune non-responsiveness to donor cells, i.e., a tolerance to the histocompatibility antigens of the donor. That is, abolishment of cytotoxic T cell activity by recipient cells against donor cells. Preferably, the recipient's T cells retain the ability to recognize other antigens as foreign, to facilitate tumor killing and general immunological responses to foreign antigens.

T cells are conditioned to become suppressor cells by treatment with a regulatory composition. A regulatory composition includes at least one compound which induces T cells to develop suppressive activity. By "'suppressive activity" herein is meant at least some of the treated T cells develop the capacity to prevent cytotoxic T cell activity in other T cells.

T cells which develop the capacity to block cytotoxic T cell activity are referred to herein as "suppressor T cells" or "regulatory T cells." By "suppressor T cells" herein is meant a population of T cells which develop the capacity to inhibit other T cells from killing donor cells.

Using methods outlined herein, graft rejection is decreased or eliminated. By "decreased" or "eliminated" herein is meant that at least one symptom of graft rejection is ameliorated. This may be

evaluated in a number of ways, including both objective and subjective factors on the part of the patient as is known in the art. The clinical pattern of organ dysfunction often helps to suggest the diagnosis of rejection. However, no clinical sign can definitively diagnose rejection. Although it would be useful to determine a means of identifying rejection episodes based on systemic manifestations of the immunologic mechanisms involved, there is not yet a well-established assay to measure rejection activity (Paul, W.E. (1999) Fundamentals of Immunology).

The methods provide for the removal of blood cells from recipients and donors. In general, peripheral blood mononuclear cells (PBMCs) are taken from recipients and donors using standard techniques. By "peripheral blood mononuclear cells" or "PBMCs" herein is meant lymphocytes (including T-cells, B-cells, NK cells, etc.) and monocytes. As outlined more fully below, it appears that the main effect of the regulatory composition is to enable CD4+ T cells and/or other T cell subsets (i.e., CD8+ T cells, NK T cells, gamma delta T cells) to develop suppressive activity. Accordingly, the PBMC population should comprise CD4+ T cells. Preferably, only PBMCs are taken, either leaving or returning red blood cells to the patient. This is done as is known in the art, for example using leukophoresis techniques. In general, a 5 to 7 liter leukopheresis step is done, which essentially removes PBMCs from a patient, returning the remaining blood components. Collection of the cell sample is preferably done in the presence of an anticoagulant such as heparin, as is known in the art.

In general, the sample comprising the PBMCs can be pretreated in a wide variety of ways. Generally, once collected, the cells can be additionally concentrated, if this was not done simultaneously with collection or to further purify and/or concentrate the cells. The cells may be washed, counted, and resuspended in buffer.

The PBMCs are generally concentrated for treatment, using standard techniques in the art. In a preferred embodiment, the leukopheresis collection step results a concentrated sample of PBMCs, in a sterile leukopak, that may contain reagents or doses of the regulatory composition, as is more fully outlined below. Generally, an additional concentration/purification step is done, such as Ficoll-Hypaque density gradient centrifugation as is known in the art.

In a preferred embodiment, the PBMCs are then washed to remove serum proteins and soluble blood components, such as autoantibodies, inhibitors, etc., using techniques well known in the art. Generally, this involves addition of physiological media or buffer, followed by centrifugation. This may be repeated as necessary. They can be resuspended in physiological media, preferably AIM-V serum free medium (Life Technologies) (since serum contains significant amounts of inhibitors of TGF-β) although buffers such as Hanks balanced salt solution (HBBS) or physiological buffered saline (PBS) can also be used.

Generally, the cells are counted; approximately from 1×10^9 to 2×10^9 white blood cells are collected from a 5-7 liter leukopheresis step. These cells are brought up in roughly 200 mls of buffer or media.

In a preferred embodiment, the PBMCs may be enriched for one or more cell types. For example, the PBMCs may be enriched for CD8+ T cells, CD4+ T cells or NK T cells. This is done as is known in

the art, as described in Gray *et al.* (1998), *J. Immunol. 160:*2248, hereby incorporated by reference. Generally, this is done using commercially available immunoabsorbent columns, or using research procedures (the PBMCs are added to a nylon wool column and the eluted, nonadherent cells are treated with antibodies to CD4, CD16, CD11b, and CD74, followed by treatment with immunomagnetic beads, leaving a population enriched for CD8+ T cells). In one embodiment, cell populations are enriched for CD4+ cells, as these appear to be the cells most useful in the methods of the invention.

In a preferred embodiment, the CD4+ cells could be further purified to include only undifferentiated, naive cells. This is done by depleting them of CD45RO+ cells using monoclonal antibodies. This procedure eliminates populations of CD4+ cells which may have acquired functions which might interfere with the generation or activity of suppressor T cells.

In other embodiments, CD8+ cells, CD3+CD4-CD8- cells, or NK T cells may be treated with a regulatory composition to develop suppressor activity.

Once the cells have undergone any necessary pretreatment, the cells are treated with a regulatory composition. By "treated" in this context herein is meant that the cells are incubated with the regulatory composition for a time period sufficient to result in T cell tolerance, particularly when transplanted into the recipient patient. The incubation will generally be under physiological temperature.

By "regulatory composition" or "tolerance composition" is meant a composition that can induce T cell tolerance to donor histocompatibility antigens. The regulatory composition will include irradiated T cell-depleted mononuclear cells from the donor to limit the suppressor T cells to only those which can react with the histocompatibility antigens of the donor and at least one compound which induces these activated T cells to become suppressor cells.

The concentration of the regulatory composition will vary depending on the identity of the compounds included in the composition, but will generally be at physiologic concentration, i.e. the concentration required to give the desired effect, i.e. an enhancement of specific types of regulatory cells.

Donor blood cells are obtained by pheresis and after removal of red blood cells, 1-2 x 10^9 PBMC are treated with anti-T-cell antibodies, such as anti-CD3 antibodies and the positive T cells removed by immuno-magnetic beads. The T cell-depleted donor cells are irradiated to prevent them from proliferating and mixed with the recipient cells at a ratio of.01 to 10, with from about 0.1 to 3 per donor cells being preferred, 0.5 to 2:1 being especially preferred, and 1:1 being ideal.

Compounds used to activate T cells to become suppressor cells include, but are not limited to, prostaglandins, nitric oxide, chemokines and cytokines. In a preferred embodiment, the compound used to activate T cells is a cytokine.

In a preferred embodiment, cytokines, such as interleukin 2 (IL-2), interleukin 15 (IL-15), interleukin 4 (IL-4), interleukin 10 (IL-10) and transforming growth factor beta (TGF-β) are used to activate T cells.

A composition containing more than one compound may be used to activate T cells to become suppressor cells. The compositions may contain more than one compound from the same class of compounds, i.e., two or more cytokines, chemokines, or prostaglandins may be mixed together. The composition also may contain compounds from different classes of compounds, such as a cytokine and a chemokine, or a cytokine and a prostaglandin, etc.

In a preferred embodiment, compositions containing two or more cytokines are used to activate T cells. For example, IL-2 and TGF-β are mixed together to increase the generation of suppressor cells.

In a preferred embodiment, TGF-β is used to generate suppressor T cells. By "transforming growth factor "β" or "TGF-β" herein is meant any one of the family of the TGF-βs, including the three isoforms TGF-β1, TGF-β2, and TGF-β3; see Massague, (1980), *J. Ann. Rev. Cell Biol 6:*597. Lymphocytes and monocytes produce the ß1 isoform of this cytokine (Kehrl *et al.* (1991), *Int J Cell Cloning 9:*438-450). The TFG-β can be any form of TFG-β that is active on the recipient cells being treated. In humans, recombinant TFG-β is currently preferred. A human TGF-β2 can be purchased from Genzyme Pharmaceuticals, Farmington, MA. In general, the concentration of TGF-β used ranges from about 10 pg to about 5 ng/ml of cell suspensions, with from about from about 0.1ng to 3 ng/ml being preferred, and 1 ng/ml being ideal.

TGF-β is incubated with the recipient cells and a population of irradiated donor PBMC (harvested as outlined above). The donor cells are irradiated so that they cannot attack the recipient cells. The incubation period occurs for a period of time sufficient to cause an effect, generally from 4 hours to 5 days although both shorter and longer periods are possible.

In a preferred embodiment, treatment of recipient cells with the regulatory composition is followed by expansion of these cells before transfer to the recipient patient.

In other embodiments, it may be desirable to transfer the cells instead of expand the cells. In this case, the cells are transferred immediately after washing to remove the regulatory composition, and frozen or otherwise stored.

Once the cells have been treated, they may be evaluated or tested prior to transplantation into the recipient. For example, a sample may be removed for: sterility testing; gram staining, microbiological studies; LAL studies; mycoplasma studies; flow cytometry to identify cell types; functional studies, etc. These and other lymphocyte studies may be done before and after treatment. A preferred analysis is to label donor cells; incubate the treated tolerant recipient cells with the labeled population to verify that the recipient cells are tolerant and will not kill the donor cells.

Assays such as those shown in Figures 2A and 2B, can also be used to determine if the regulatory composition has induced suppressor cells. This is done by mixing suppressor cells generated ex vivo with the recipient's T cells and assaying the survival of the donor cells.

In a preferred embodiment, the treatment results in the conditioning of the T cells to become non-responsive to histocompatibility cells of the donor so that graft rejection is prevented.

In a preferred embodiment, the isolation of T cells, conditioning them with the regulatory composition, and expansion of these cells are performed in a closed system such as the Nexell Isolex 300 system to minimize the introduction of toxins to the product.

The recipient's treated T cells are transferred back to the recipient patient. This is generally done as is known in the art, and usually comprises injecting or introducing the treated cells into the recipient as will be appreciated by those in the art. This may be done via intravascular (IV) administration, including intravenous or intraarterial administration, intraperitoneal administration, etc. For example, the cells may be placed in a 50 ml Fenwall infusion bag by injection using sterile syringes or other sterile transfer mechanisms. The cells can then be immediately infused via IV administration over a period of time, such as 15 minutes, into a free flow IV line into the patient. In some embodiments, additional reagents such as buffers or salts may be added as well.

After reintroducing the cells into the patient, the effect of the treatment may be evaluated, if desired, as is generally outlined above and known in the art.

The following examples serve to more fully describe the manner of using the above-described invention, as well as to set forth the best modes contemplated for carrying out various aspects of the invention. It is understood that these examples in no way serve to limit the true scope of this invention, but rather are presented for illustrative purposes. All references cited herein are incorporated by reference in their entirety.

EXAMPLES

Example 1

TGF-β activated CD4+ T cells suppress cytotoxic T cell activity.

Blood from both donor and recipient were obtained by pheresis. PBMC from each individual were separated from RBC using the Nexell Isolex 500 closed system. CD4+ cells from the recipient and T cell-depleted mononuclear cells from the donor were prepared using commercially available reagents.

If the recipient receives an organ transplant from a donor whose mononuclear cells are not available, the recipient's T cells are conditioned with irradiated mononuclear cells from a pool of donors which express a broad panel of common and uncommon histocompatibility antigens.

Recipient CD4+ cells were cultured with irradiated donor mononuclear cells in the presence of TGF-β for 5 days. The CD4+ cells which react with donor cells in the presence of TGF-β were induced to become suppressor T cells. The cells were incubated with donor alloantigens or mitogens for an additional 10 days to expand the number of suppressor T cells.

To test the potency of these suppressor T cells, they were cultured for 5 days with the recipient's T cells mixed with donor irradiated non-T cells to induce killer T cells. The recipients T cells are then mixed with chromium labeled donor lymphoblast cells and incubated for 4 hours. If the donor cells are killed by the recipients cytotoxic T cells, chromium is released into the culture medium. By determining the amount of chromium released, the percentage of cells killed can be determined. Figures 2A-4B show that regulatory T cells generated with TGF-beta block the ability of the recipient's T cells to kill donor T cells.

In the standard cytotoxic assays shown in Figures 2A-4B, recipient cells were cultured with labeled donor cells in 25:1, 50:1 and 100:1 ratios. These combinations of recipient and donor cells are called effector to target ratios. Killing is indicated by the various symbols. As expected, maximum killing was seen at the highest effector to target ratio. CD4+ cells cultured with donor stimulator cells had a modest inhibitory effect. CD4+ cells that had been conditioned with TGF-β 1 ng/ml almost completely abolished the capacity of recipient T cells to kill donor mononuclear cells.

Example 2

TGF-β activated T cells suppress cytotoxic T cell activity.

Blood from both donor and recipient were obtained by pheresis. PBMC from each individual were separated from RBC using the Nexell Isolex 500 closed system. T cells from the recipient which contain both the major subsets (CD4+ and CD8+ cells) and the minor subsets (TNK cells and gamma delta T cells), and T cell-depleted mononuclear cells from the donor were prepared using commercially available reagents.

Recipient T cells were cultured with irradiated donor mononuclear cells in the presence of TGF-β for 3 to 5 days. The various T cell subsets which react with donor cells in the presence of TGF-β were induced to become suppressor T cells. Similar procedures were repeated for an additional 10 days to expand the number of suppressor T cells.

The recipient's T cells primed with donor alloantigens in the presence of TGF-beta are then tested for suppressive activity by showing that they prevent recipient's precursor killer cells from developing the capacity to kill donor T cells by the procedures described in Example 1.

Example 3

Treatment of T cells from a recipient of kidney graft
from a non-identical sibling donor to prevent graft rejection.

CD4+ cells conditioned with 1 ng/ml TGF-β, are transferred to the recipient 1 day before kidney transplant and allowed to "home" to lymphoid tissue. These CD4+ cells circulate to the recipient's lymphoid organs, where they block the recipient's T cell response to donor histocompatibility antigens. As a result, the recipient's T cells become tolerant to the donor's histocompatibility antigens. This tolerance reduces acute rejection, lessening the need for high doses of immunosuppressive drugs. As the recipient's lymphocytes are "educated" to develop long lasting tolerance, chronic rejection is

decreased or eliminated. If signs of graft rejection recur, additional infusions of regulatory T cells will ameliorate this response.